Microhardness changes in dentine after neonatal capsaicin application

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Abstract

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Aim To determine if desensitization of the nociceptive innervation in the dental pulp has an effect on odontoblast function in the rat.

Methodology Neonatal systemic application of capsaicin was used to selectively eliminate nociceptive innervation. 12 capsaicin-treated rats were intravitally perfused at 150 days of life with 4% formaldehyde and jaws were prepared for Vicker's microhardness (VMH) measurement. As a control, 12 rats were injected with vehicle on the 3rd day of life and intravital perfusion was carried out exactly as those used for the experimental group. Immunohistological labeling of CGRP was carried out in both groups to assure the efficiency of desensitization in the experimental group. The VMH was measured in the incisors of each animal for a quantitative analysis of dentine quality.

Results Vicker's microhardness was significantly higher in the control rats compared with the capsaicin-treated rats ($P < 0.001$).

Conclusions Neonatal systemic application of capsaicin produces changes in the quality of dentine in the rat over time and therefore it is suggestive that selective elimination of the nociceptive innervation in pulpal tissue may effect odontoblast function.

Keywords: dentin, hardness, neuronal, odontoblast, pulp.

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Introduction

The odontoblast–neuronal relationship has been of great interest in the literature, but it is unclear which mechanisms and influences this relationship has on pulpal physiology. It has been established that sensory nerves densely innervate oral tissue, supplying their peripheral tissue with neuropeptides of the tachykinin family and Calcitonin Gene Related Peptide (CGRP) (Olgart *et al.* 1977, Wakisaka *et al.* 1985, Byers *et al.* 1987, Casasco *et al.* 1990, Luthman *et al.* 1992). Furthermore, it has been concluded that specific neuronal receptors (neurokinin-1) are present on odontoblast cell bodies within pulpal tissue as well as odontoblast processes within dentine (Fristad *et al.* 2002). It has been established in a previous study that the neonatal systemic desensitization with capsaicin has profound effects on tooth development (Raab *et al.* 1996). Raab *et al.* (Raab *et al.* 1996) reported that there is a three-fold reduction in the thickness of the pre-dentinal layer after neonatal application of capsaicin. Furthermore, they demonstrated that the elimination of the neuronal innervation in the pulp has detrimental effects on dentine production, producing concavities in dentine that were detectable with SEM (Raab *et al.* 1996).

Capsaicin (8-methyl-*N*-vanillyl-6-nonenamide), the pungent ingredient in chili peppers, has been shown to produce a selective desensitization of nociceptive fibres when applied to a physiological system neonatally and systemically by eliciting a response specifically to C fibres

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and A-δ fibres (Lawson 1987). When topically applied to the skin and mucosa, capsaicin produces a local desensitization that recovers after a few days (Szolcsanyl *et al.* 1990, Maggi 1991). This is irreversible when applied systemically at the neonatal phase of life (Szolcsanyl *et al.* 1990, Maggi 1991). It has been further established that this irreversible desensitization is produced by an excessive calcium influx in the cell body, which, in turn, blocks the conduction of action potentials of the neuron (Maggi 1991). Furthermore, others have established that the neonatal systemic application of capsaicin induces an estimated functional loss of 89% of the unmyelinated C-fibres and approximately 36% of the myelinated A fibres (Lawson 1987). Therefore, the neonatal systemic application of capsaicin in the rat produces a nociceptive desensitized animal model in which it is possible to analyse the physiological consequence of nociceptive inactivation in the dental pulp.

The purpose of this study was to determine what influences neonatal treatment with capsaicin has on odontoblast function and dentine development by quantitatively analysing dentine microhardness in nociceptive-desensitized rats compared with nociceptive-intact rats after 150 days of life.

Material and methods

This project was carried out according to the standards of the local animal research and ethics committee. Twelve Wistar rats received a subcutaneous injection of 50 mg kg^{-1} body weight 1% capsaicin sterile solution (in vehicle containing 10% ethanol, 10% Tween 80 and 80% NaCl saline) on the third post-natal day. A group of 12 rats injected with vehicle alone of the same volume on the third post-natal day served as a control group. All animals from both groups were anaesthetized and sacrificed by intravital perfusion and fixation with 4% paraformaldehyde at day 150. Jaws were dissected from all animals and further investigation was carried out with measurement of VMH.

The effectiveness of desensitization was controlled by immunohistological analysis of adjacent incisors in each animal for CGRP. Immunohistological labeling was carried out with horizontal cryosections of incisors at 30 μm using a Vectastain pK4001-kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's instructions with some modifications. Briefly, sections were washed in 0.01 mol PBS and incubated with 0.3% hydrogen peroxide in PBS for 30 min at +4 °C. After PBS washes, the sections were incubated with normal goat serum in PBS for 1 h at room temperature. Subsequently, sections were incubated with rabbit anti-CGRP serum (1:10:000; Sigma, Saint Louis, MO, USA) diluted in PBS containing 1% bovine serum albumin (Sigma) for 72 h at +4 °C. A negative control of the immunohistological reaction was carried out by incubating sections where the primary antibody was omitted. After PBS washes, biotinylated goat secondary antibodies against rabbit IgG (Vector Laboratories) were applied onto the sections for 1.5 h at room temperature. After further PBS washes, the sections were incubated for 4 h with avidin–biotinperoxidase complex. Final visualization was made with 0.2% nickel-enhanced 0.05% diaminobenzidine (Sigma) and 0.003% hydrogen peroxide. Sections were then mounted onto gelatin-coated slides and counterstained. Image analysis of this immunohistological labeling was carried out with Scion Image, in which the threshold of the CGRP-labeling was defined for each subject and the number of pixels of this defined threshold was measured per square inch within a defined region of pulp. The selected region of threshold measurement was consistent between subjects. Statistical differences were determined using SPSS 11.5 to calculate Student's *t*-test.

The VMH measurement procedures were carried out using the MHT-10 V microhardness tester from Zeiss (Jena, Germany). With this device, a diamond in the shape of a square-based pyramid was pressed into the polished surface of the incisor dentine under a load of 490.33 N (50.00 kgf mm^{-2}). The VMH was measured in the incisors of each animal from each group. Measurements were taken across the diameter of the incisor at three different regions of the incisor. These regions were located at the incisal 1/3, the middle 1/3, and the apical 1/3 of the tooth. At each of these measurement points on the incisor, the measurements were taken across the diameter of the incisor at 15 points in intervals of 200 μm, starting at −100 μm from the dento-enamel junction (DEJ) on the convex side and ending −100 μm from the dentine border (DB) on the concave side (Fig. 1). Overall mean values for VMH were calculated in the experimental and control group. SPSS 11.5 was applied to calculate mean values at each incisor region (incisal 1/3, middle 1/3, apical 1/3) for the experimental and control rat group and the Student's *t*-test was used to determine significant differences between study groups.

Results

Immunohistological analysis demonstrated that there was a significant reduction ($P < 0.05$) in sensory

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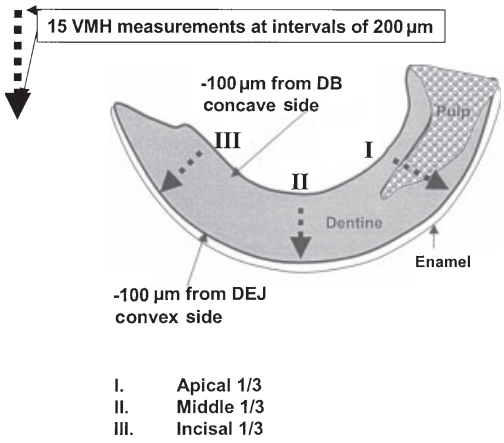

Figure 1 There were three regions of measurement on the rat incisor (apical, middle and incisal 1/3). At each region of measurement, 15 points of measurement were carried out across the diameter of the incisor (intervals of 200 µm) starting at −100 µm from the DEJ on the convex side and ending at −100 µm from the DB on the concave side.

innervation in the capsaicin-treated rat. While the mean number of pixels inch^{-2} identifying CGRP labeling was 0.0106 (SD: 0.0104) for the capsaicin-treated group, the control group demonstrated a mean of 0.05820 pixels inch^{-2} (SD:0.0778) ($P = 0.047$). Photographic documentation of this difference is presented in Fig. 2.

While the overall mean VMH was measured at 73.33 Vickers Hardness Number (VHN) (SD: 9.49) for the control rat group, the experimental rat group demonstrated an overall mean of 67.10 VHN (SD: 9.23). The Student *t*-test showed a significant difference between the two study groups ($P < 0.001$). Also, these differences in VMH between the experimental group and the control group were continuous at two (incisal 1/3 and middle 1/3) of the three measured regions of the incisor. In the apical 1/3 measured region, VMH was similar for both groups at measurements approaching the pulp chamber (Fig. 3).

Discussion

Vicker's microhardness is suitable for determining the hardness of very brittle material, such as dental hard tissue (Lysaght & Debellis 1969). This study demonstrated a significant quantitative difference in the VMH of dentine in the incisors between the capsaicin-treated rats and the control rats. The difference in VMH suggests that a disturbance in dentine apposition is present in the neonatally desensitized rat. It is likely that this difference is due to an effect on the dentine matrix apposition from odontoblast processes when nociceptive neuropeptides are absent from the odontoblast cell body environment. It has been reported in the literature that trauma and noxious stimuli to the dental pulp produce a dentine matrix response from the odontoblasts (Klinge 2001). Furthermore, the differences seen in VMH could also be due to a difference in the mineralization rate of the dentine matrix between the two study groups. It has been demonstrated that the odontoblast plays a key role in this process by transcellular ion transport (Lundquist 2002). While the differences in VMH were continuous at the incisal and middle 1/3 of the incisor between both study groups, the measurements for VMH were identical for both groups at the dentine region directly adjacent to the pulpal chamber. This suggests that differences observed are likely due to changes in the mineralization process taking place at the pre-dentine–dentine interface. While capsaicin is a neurotoxin and odontoblasts are of neural crest origin, one must also consider the possibility of a direct toxic effect on odontoblast and therefore future work should include comparative studies with other pulpal denervation models, which do not use capsaicin.

In the present study, a difference in quality of dentine structure was found between the capsaicin-treated rat group and the control rat group after 150 days of life.

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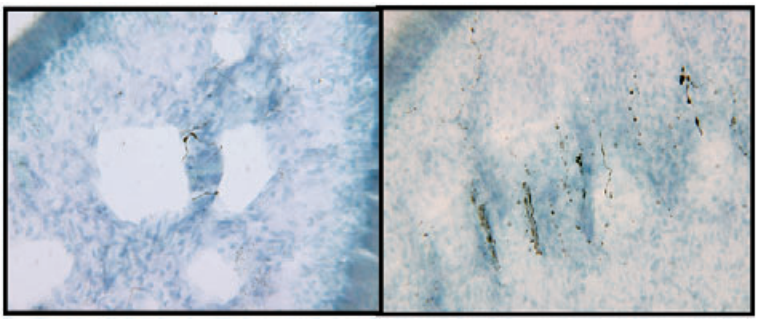

Figure 2 Photographic documentation of reduction in CGRP neuropeptide labeling in capsaicin-treated rats (left) compared with control rats (right) treated with vehicle alone. In 12 animals from each group measured, a significant difference ($P < 0.05$) in pixels representing CGRP-labeling inch^{-2} was determined between study groups.

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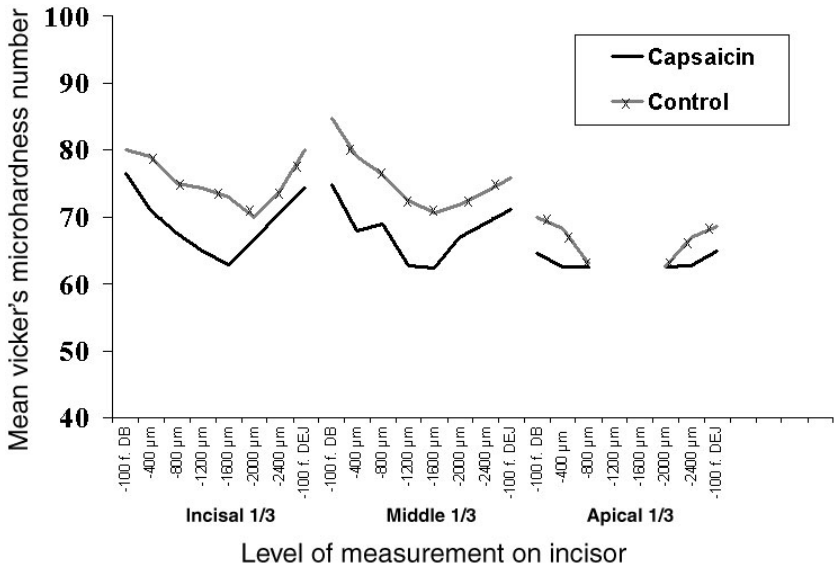

Figure 3 Mean Vicker's Hardness Number for the experimental group (capsaicin) and control group (control) at three defined regions on the rat incisor. **Missing data points in the central region of the apical 1/3 portion of the graph present the pulpal chamber of this region.

The changes observed in dentine from the capsaicin-treated rat may be interpreted as signs of physiologically malfunctioning odontoblasts. However, it is still unclear if this influence is a direct physiological effect between the cell–cell interaction of the odontoblast and nociceptive neuron, a neurotoxic effect of capsaicin directly on odontoblasts, or if these effects are due to an indirect physiological effect such as neuropeptide influences on pulpal microcirculation. In the literature, it has been questioned if a synaptic relationship exists between the odontoblast and its pulpal fibres (Inoue *et al.* 1995). This question, however, is still unresolved (Tsukada 1987, Norlin *et al.* 1999). Furthermore, it is known that nociceptive neuropeptides such as CGRP and Substance P (SP) promote wound healing upon the exposure to a painful stimulus by triggering a vasodilatory response (Raab *et al.* 1988, Kim 1990, Olgart *et al.* 1991, Byers 1993). Therefore, in the absence or reduction of CGRP and SP release in the dental pulp of the neonatally desensitized capsaicin-treated rat, a reduction in microcirculation will occur and may have an indirect effect on odontoblast function (Lui *et al.* 1992).

Conclusion

The present observations in the VMH analysis of incisor dentine lead to the conclusion that neonatal systemic application of capsaicin significantly influences the quality of dentine produced by odontoblasts in the rat. The exact physiological mechanism for this influence between odontoblast cell bodies and nociceptive fibres in the pulp is still unclear and further investigation is necessary to understand this cell–cell interaction.

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